Acid Lability of Rhinovirus Type 14: Effect of pH, Time, and Temperature (37634)

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(Introduced by H. G. Cramblett)

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The picornavirus group of viruses are divided into four subgroups; enteroviruses, cardioviruses, rhinoviruses, and foot-and-mouth disease viruses (7). Foot-and-mouth disease viruses (FMDV) and rhinoviruses (RV) are differentiated from other picornaviruses on the basis of their acid lability in the absence of halide ions, and by their greater buoyant densities in solutions of cesium chloride (CsCl).

The enteroviruses, polio-, echo-, and coxsackie-viruses, are acid stable. The cardioviruses, Maus-Elberfeld (ME), encephalomyocarditis (EMC), and Mengo, which are chemically and physically indistinguishable from enteroviruses, can be either acid labile or stable depending on environmental conditions. At pH 5.0–7.0 in solutions of 0.1 *M* chloride or bromide ions, ME-virus is rapidly inactivated. This inactivation is dependent not only upon the presence of certain halide ions, but also on temperature and ionic strength (17). Mengovirus behaves similarly (12, 18).

Tyrrell and co-workers (19, 20) and Hitchcock and Tyrrell (8) observed that rhinovirus type 2 lost its ability to produce a cytopathic effect, interference, or colds in humans after treatment at pH 2.0–2.1. Ketler *et al.* (9) reported the loss of rhinovirus infectivity after attempts to concentrate these viruses by precipitation at low pH. Since then other reports on the loss of infectivity following acid treatment of rhinoviruses have appeared in the literature (5, 6, 15, 16). The reason(s) for the extreme sensitivity of the rhinovirion to acid conditions is not known. Little information is available on the mechanism(s), kinetics, effect of temperature or other events leading to the loss of infectivity following acid treatment of the rhinovirion. The only recent physiocochemical observations on the acid lability of rhinoviruses have been reported by Medappa *et al.* (13) and Korant *et al.* (10). Medappa and co-workers found that after acid treatment most of the viral proteins had aggregated. Korant and associates found a loss of rhinovirus polypeptide 4 (VP4) and the production of "A" and "B" type particles following treatment at pH 5.0.

The purpose of this report is to describe the effects of acid treatment on rhinovirus type 14. Experiments involving acid inactivation as a function of pH, time, and temperature are reported.

Materials and Methods. Viruses and cell culture. Two picornaviruses were used in these studies; human rhinovirus type 14 (HRV-14), strain 1059, and poliovirus type 2, Sabin. Both viruses were propagated in cultures of HeLa cells. The procedures for growth and maintenance of HeLa cells have been reported elsewhere (3).

For viral propagation, monolayer cultures were infected by adding virus directly onto cell cultures previously drained of their tissue culture medium. Following adsorption for 2 hr at 33° for rhinovirus or 37° for poliovirus, 40–60 ml of maintenance medium were added to the culture bottles. Cultures infected with rhinovirus were incubated at 33° on a rocking platform (Model 6700, Bellco Glass, Inc., Vineland, New Jersey) at 3–6 cpm. Poliovirus-infected cultures were incubated at 37° in a stationary position. When cytopathic effects (CPE) were complete, the cultures were stored at —20° until used.

Viruses were quantitated by either infectivity titrations in roller tube cultures or by plaque assay as described by Conant *et al.* (4).

Purification of viruses. Infected cultures were frozen (—20°) and thawed three times and clarified by centrifugation at 400*g* for 10 min. The fluids were mixed with an equal volume of trichlorotrifluorethane (Freon 113, E. I. DuPont de Nemours Company, Wilmington, Delaware) and blended at maximum speed for three 1-min intervals in a Sorvall omni-mixer (Ivan Sorvall, Inc., Norwalk, Connecticut) submerged in an ice bath. Following homogenization, the mixture was clarified by centrifugation at 900*g* for 10 min and the aqueous fractions containing virus were stored at —30°. Prior to concentration, the viral preparations were clarified again by centrifugation at 900*g* for 10 min. The virions were then sedimented by centrifugation in a No. 30 fixed-angle rotor at 30,000 rpm (105,165*g*) for 4 hr at 5° in a Model L Spinco preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California). The viral pellets were resuspended in 0.01 *M* Tris buffer (tris-hydroxymethyl-aminomethane) pH 8.1 and subjected to cesium chloride isopycnic centrifugation in a SW 39 rotor at 39,000 rpm (175,296*g*) for 48–60 hr. Fractions from each gradient were collected dropwise through a hole punctured in the bottom of each centrifuge tube. CsCl was removed by dialysis against 0.01 *M* Tris buffer (pH 8.1).

Virus inactivation. Inactivation of HRV-14 as a function of pH was determined in the following manner. Approximately 2×10^7–2×10^8 PFU/ml were diluted 1:10 (final volume 2.0 ml) in 9 different citrate-phosphate buffers ranging in pH from 3.0–8.0. The initial molarity for the citrate-phosphate was 0.1 *M* and 0.2 *M*, respectively. After 2-hr incubation at room temperature, the residual infectivity was determined by plaque or tube assay.

For kinetic and temperature studies, generally 0.2 ml of HRV-14 containing between 5×10^6–6×10^7 PFU/ml was incubated with 0.8 ml of pH 3.0 or 5.0 citrate-phosphate buffer or Eagle's minimum essential medium (MEM) adjusted to pH 2.9–3.0 with 1.0 *M* Tris base. MEM was prepared without salts or sodium bicarbonate. The final pH's after adding virus were 3.0 and 5.0. At the desired time intervals, inactivation was stopped by adding 1.0 ml of pH 8.0 citrate-phosphate buffer. The amount of residual infectivity was determined by plaque assay. Residual virus was passed once in cell culture and retested for acid inactivation.

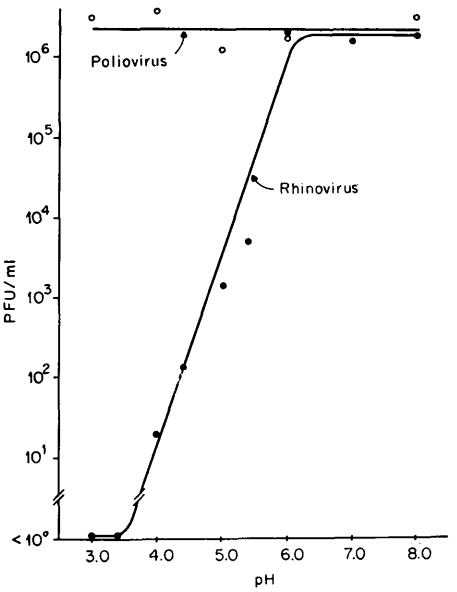

FIG 1. Inactivation of human rhinovirus type 14 as a function of pH. Viral samples were diluted 1:10 in citrate-phosphate buffers ranging in pH from 3.0–8.0. After incubation for 2 hr at room temperature, the amount of residual virus was determined by plaque assay. The total volume was plated from all the buffers used below pH 5.0. Poliovirus was included as an example of an acid stable picornavirus and was handled like the rhinovirus. The viral concentrations (PFU/ml) at time zero and after 2 hr for poliovirus were 2.5×10^6 and 2.0×10^6, respectively. The corresponding rhinovirus concentrations were 2.2×10^6 and 1.8×10^6.

Results. Effect of pH. Purified HRV-14 and poliovirus type 2 were diluted 1:10 in 0.2 *M* citrate-phosphate buffers with pH values of 3.0, 3.4, 4.0, 4.4, 5.0, 5.4, 6.0, 7.0, and 8.0. Rhinovirus cell lysates clarified by centrifugation were treated similarly, except at pH 3.0 and 3.4—the samples were diluted 1:2 instead of 1:10.

Figure 1 shows the inactivation of purified rhinovirus as a function of pH after incuba-

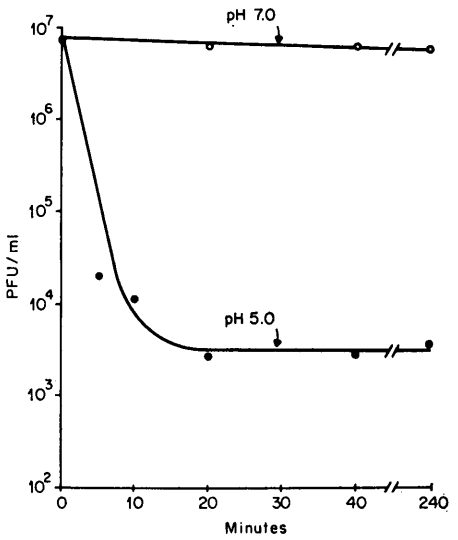

FIG. 2. Inactivation of human rhinovirus type 14 at pH 5.0 as a function of time. Purified HRV-14 samples were placed in pH 5.0 citrate-phosphate buffer at 24°. At specified time intervals, the reaction was stopped by diluting aliquots 1:5 with pH 8.0 citrate-phosphate buffer. The amount of infectious virus present was determined by plaque assay. The initial virus concentration was between 5×10^6–6×10^7 PFU/ml.

tion at room temperature for 2 hr. Between pH 6.0–8.0 no loss of infectivity was observed. Treatment at pH 5.4 resulted in a loss of more than 99% of the infectivity. Maximum inactivation occurred at pH 3.4 and 3.0. Infectious virus was present after 2 hr at all pH values above 3.4. No virus was detected at pH values lower than 3.4. Similar results were obtained with unpurified (clarified) rhinovirus preparations. There was no significant loss of poliovirus infectivity at any pH.

To determine the effect of time on inactivation of human rhinovirus type 14, kinetic experiments were done at pH 3.0 and 5.0. Inactivation was initiated by making a 1:2 dilution of HRV-14 in MEM (pH 3.0) or citrate-phosphate buffer (pH 5.0). To stop inactivation, the pH of the reaction mixture was raised above 6.0 by diluting 1:5 with pH 8.0 citrate-phosphate buffer. Figure 2 shows a typical inactivation curve of a purified preparation of HRV-14 as a function of time. There was no loss of infectivity at pH 7.0. At pH 5.0, infectivity losses greater than 99% were observed. Inactivation reached a maximum by 20 min, and leveled off thereafter yielding a surviving fraction. When unpurified (clarified) virus preparations were used for kinetic studies at pH 5.0, the rate of inactivation was similar to that observed for purified viral preparations.

Experiments performed to determine the effect of pH 3.0 treatment on virus surviving previous exposure to pH 5.0 showed that lowering the pH from 5.0 to 3.0 completely eliminated the surviving fraction (Table I). Passage of the surviving fraction in cell culture did not yield a more resistant population of virions (unpublished observations).

Attempts to perform kinetic experiments at pH 3.0 were unsuccessful. Inactivation was extremely rapid and no infectious virus could be detected after 10 sec of incubation (Table II).

Effect of temperature. Experiments involving the effect of temperature on the inactivation of HRV-14 at pH 5.0 revealed that inactivation was temperature dependent (Table III). Virus incubated at 0° for 5 min in pH 5.0 citrate-phosphate buffer showed no loss of infectivity. In contrast, virus incubated for 5 min at 24° in the same buffer lost nearly 99% of its infectivity. At

TABLE I. Loss of Infectivity of Human Rhinovirus Type 14 After pH Change from 5.0 to 3.0.

	Surviving virus (PFU/ml)		
Time (hr)	pH 8.0[a]	pH 5.0[b]	pH 5.0 to pH 3.0[b]
0	1.3×10^7	—[c]	—[c]
1	—[c]	3.0×10^4	3.0×10^4
2	9.6×10^6	2.8×10^4	$<1.0 \times 10^1$

[a] Virus was plaque assayed at time zero and then again after dialysis for 2 hr at room temperature against pH 8.0 citrate-phosphate buffer.

[b] A viral sample was divided into two aliquots; one aliquot was plaque assayed after dialysis for 1 hr at room temperature against pH 5.0 citrate-phosphate buffer. The second aliquot was dialyzed for 1 hr at room temperature against pH 5.0 citrate-phosphate buffer, followed by dialysis for 1 hr at pH 3.0, and then plaque assayed for infectious virus.

[c] Not tested.

TABLE II. Loss of Infectivity of Human Rhinovirus Type 14 at pH 3.0.

Time (sec)	Surviving virus (PFU/ml)	
	pH 8.0[a]	pH 3.0[b]
0	2.8×10^7	—[c]
10	—[c]	$<1.0 \times 10^1$
300	2.5×10^7	$<1.0 \times 10^1$

[a] Rhinovirus type 14 was incubated in citrate-phosphate buffer at room temperature.

[b] Eagle's minimal essential medium (MEM) was adjusted to pH 3.0 with 1.0 *M* Tris base, and used for inactivation studies. Poliovirus incubated at pH 3.0 in MEM for 3 hr at room temperature showed no significant loss of infectivity. The loss of rhinovirus infectivity was pH dependent and not menstruum dependent.

[c] Not tested.

pH 3.2, no infectious virus was detected after incubation at 0° for 5 min (Table III).

Discussion. An important characteristic of rhinoviruses is loss of infectivity at low pH (5, 6, 9). However, there is no information on the interrelationships of pH, time, and temperature. Ketler *et al.* (9) tested 28 distinct rhinoviruses and reported that no infectious virus survived at pH 3.0 after 2-hr incubation at room temperature. Dimmock and Tyrrell (6) found that 7 different human rhinovirus strains lost from 1.3–3.0 $\log_{10}$ of infectivity after incubation at pH 5.0–5.4 in phosphate buffer for 60 min at 37°. With four other rhinoviruses, these same investigators found greater than 2.1, 2.6, 3.8, and 4.5 $\log_{10}$ reduction in infectivity at pH 4.3–4.5.

In our study, rhinovirus infectivity survived treatment at pH 5.0 but not at pH 3.0. At pH 5.0, inactivation reached a maximum after 20 min and the amount of residual infectious virus remained constant for up to 2-hr incubation. The explanation for this surviving fraction is not apparent. The surviving fraction probably is not due to reactivation of virus since there were no inflections in the kinetic curves after adjusting the pH to stop inactivation. Possibly, the surviving fraction is the result of viral aggregation at pH 5.0. Virions in the center of aggregates might be protected from the acid environment. The destruction of this surviving fraction at pH 3.0 could be due to dissociation of aggregates resulting in a complete loss of infectivity at the lower pH. No attempts were made to test for the presence of aggregated virus.

The possibility that some virions are more resistant than others to inactivation at pH 5.0 cannot be excluded. However, attempts to select out such virions were unsuccessful. This suggests that survival at pH 5.0 may be related to environmental conditions and not necessarily to genetic differences. The possibility of the presence of infectious nucleic acid was not explored.

Acid inactivation of rhinovirus appears to be similar in some respects to the acid inactivation of FMDV, but FMDV appears to be more acid labile than rhinoviruses. Bachrach *et al.* (1) reported that acid inactivation of FMDV at pH 5.0 or 6.0 (4°) resulted in a surviving fraction. This fraction was much less at pH 5.0 than reported here for HRV-14. Furthermore, at both pH 5.0 and 6.0, essentially all infectivity of FMDV was lost in 10 sec and 10 min, respectively. In contrast, HRV-14 did not lose infectivity at pH 6.0 and 24° even after 2-hr incubation (Fig. 1).

At 0°, no loss of rhinovirus infectivity

TABLE III. Effect of Temperature on Infectivity of Human Rhinovirus Type 14 at pH 5.0 and 3.0.

Temperature	pH 7.0[a] (PFU/ml)	pH 5.0[b] (PFU/ml)	pH 7.0[a] ($TCID_{50}$/ml)	pH 3.2[c] ($TCID_{50}$/ml)
0°	3.8×10^7	3.9×10^7	10^8	$\leq 10^{.5}$
24°	3.2×10^7	3.8×10^5	10^8	$\leq 10^{.5}$

[a] Initial viral concentration in pH 7.0 citrate-phosphate buffer at 0° and 24° at time zero.

[b] Virus was incubated in pH 5.0 citrate-phosphate buffer for 5 min at 0° and 24°.

[c] Virus was incubated in pH 3.2 MEM for 5 min at 0° and 24°.

was observed after treatment at pH 5.0 for 5 min. A temperature dependence on inactivation in the presence of certain halide ions has been reported for cardioviruses. ME-virus did not lose any infectivity after treatment at pH 5.7 for 10 min at 0°; while at 37°, greater than 90% of the infectivity was lost (17). At 7°, Mengovirus lost less than 10% infectivity after 90 min of treatment at pH 6.2; while at 37°, greater than 99.9% infectivity was lost over the same time interval (12).

Although the experimental conditions have varied, the acid lability characteristics of caliciviruses (feline picornaviruses) appear to differ from rhinoviruses and FMDV. Burki and Pichler have reported (2) that caliciviruses are relatively stable at pH 3.0. Even at pH 2.2, the infectivity of five out of six distinct feline viruses was not completely destroyed. Lee and Gillespie (11) also reported that the infectivity of two different caliciviruses survived treatment at pH 3.0 after 15 min of incubation at room temperature.

Recently, Newman *et al.* (15) suggested classifying mammalian picornaviruses into six subgroups on the basis of buoyant densities in cesium chloride and on base compositions of nucleic acids. From the literature and our results, it appears that the picornaviruses can now be possibly segregated into at least five subgroups on the basis of characteristic reactions at low pH. Enteroviruses are unique because of stability at pH 3.0. In contrast to rhinoviruses, FMDV is inactivated at pH's as high as 6.5 and at low temperatures at pH 5.0 and 6.0. The caliciviruses can be differentiated from rhinoviruses and FMDV on the basis of greater stability at low pH and kinetics of inactivation. For cardiovirus, inactivation at low pH is dependent upon the ionic strength of certain halide ions in the menstruum. Thus, the single parameter of acid lability, or acid lability in conjunction with, temperature of inactivation, kinetics of inactivation, or ionic strength may be a relatively simple and rapid means for classifying picornaviruses.

Summary. The kinetics of acid inactivation of human rhinovirus type 14 are affected by pH and temperature of incubation.

At pH 3.0, inactivation was complete within 10 sec with total loss of infectivity. At pH 5.0, inactivation reached a maximum by 20 min and yielded a persistent fraction of infectious virus. This surviving fraction was eliminated when the pH was lowered to 3.0. At 0° no loss of infectivity was observed after 5 min of incubation at pH 5.0, while at room temperature 99% of the infectivity was lost. The characteristics of rhinovirus inactivation at low pH appears to be different from all other picornaviruses.

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